LONG-TERM GROWTH *IN VITRO* OF ISOLATED, FULLY DIFFERENTIATED NEURONES FROM THE CENTRAL NERVOUS SYSTEM OF AN ADULT INSECT

By E. A. HOWES, T. R. CHEEK AND P. J. S. SMITH

Laboratory of Molecular Signalling, Department of Zoology, Downing Street, Cambridge, UK

Summary

A method is described for the isolation and growth *in vitro* of fully differentiated neurones from the thoracic ganglia of adult cockroaches. The presence of insect blood in the culture system is shown to promote growth. The morphology of the growing neurones and the plasticity of the branching processes are described and growth rates are measured. Using a fluorescent Ca^{2+} indicator dye, changes of intracellular calcium levels in the growing neurones in response to K^+ depolarization have been measured. The results, indicating the presence of voltage-dependent Ca^{2+} channels on neuronal processes *in vitro*, show that neurones can be maintained in a functional state for several weeks by this technique. Such preparations could prove useful for studying a variety of physiological and pharmacological properties of neurones, including the mechanisms controlling growth, synapse formation and neuronal interactions with other cell types.

Introduction

Insect central nervous systems (CNS) have provided versatile preparations for the study of many aspects of neurophysiology and pharmacology. Among the strengths of the insect CNS for this type of research are its accessibility in both the adult and embryonic stages, its relative simplicity compared with vertebrate systems, its ability to survive experimental procedures and its plasticity in regeneration. These factors, together with the economic importance of many insects and the need to study insecticidal action at the level of the CNS, have led to the accumulation of a large body of information about insect neural function. Further work, particularly in the field of neuronal growth and phenotypic plasticity would be greatly facilitated if experiments could be carried out on individual elements in isolation from the rest of the nervous system. To this end, a number of workers have developed techniques for culturing neural cells from the fully differentiated CNS of adult invertebrates. Considerable success has been achieved

with some taxa, notably annelids (Ready and Nicholls, 1979), molluscs (Dagan and Levitan, 1981) and crustaceans (Schacher and Proshansky, 1983; Graf and Cooke, 1990), but adult insects have been less amenable to this approach.

Embryonic insect nervous systems have proved more tractable. As long ago as 1970, Chen and Levi-Montalcini described a system for the growth and maintenance of embryonic cockroach neurones *in vitro*. Since then a number of laboratories pursuing this line of research (reviewed by Beadle and Hicks, 1985; Thomas *et al.* 1987) have succeeded in obtaining long-term cultures of neurones and glial cells from the embryonic CNS of cockroaches, locusts and flies. Nymphal and larval tissues have also been used to produce dissociated neurones in cell culture. Wu *et al.* (1983) developed a technique for growing primary dissociated neurone cultures from *Drosophila melanogaster*. These were obtained from third-instar larval CNS, at a period when the CNS is undergoing considerable development (White and Kankel, 1978). In heterometabolous insects, nymphal neuronal cultures can be obtained from third-instar locusts, provided they are co-cultured with established embryonic cells (Giles and Usherwood, 1985). In adults, neurosecretory cells from the brain of cockroaches have been grown *in vitro* (Seshan and Levi-Montalcini, 1973), but this work was not extended to other neural tissue. Short-term *in vitro* experiments have been carried out on neurones from adult cockroaches and houseflies (Pinnock and Sattelle, 1987), but these survived for only a few days and showed little sign of outgrowth. Recently, growth of neurones from adult *Musca domestica* has been described (Harrison *et al.* 1990). In mixed cultures of glial and neuronal cells derived from fly brain some sprouting of neurones was observed, usually restricted to 'a few cell body diameters' (cell body diameter being 5–15 μm), with some 50 % of cells remaining viable for at least 7 days. However, in general, the initiation and long-term maintenance of substantial growth in neurones isolated from the adult insect CNS has not been achieved.

Studies on the repair processes following glial lesions of adult cockroach CNS have shown that both perineurial and subperineurial glial cells proliferate *in vivo* (Smith *et al.* 1990) and that blood cells play an important part in the initial stages of repair (Smith *et al.* 1986; Howes *et al.* 1987). Some aspects of the cellular reactions involved in repair are difficult to study *in vivo*, and it was decided to develop an *in vitro* system in which cellular reactions could be observed in controlled conditions. Recently, we succeeded in showing that a reactive glial cell class derived from the CNS of adult cockroaches could be grown *in vitro* for periods of more than 6 weeks (Howes *et al.* 1989). Continued work on this preparation has shown that the presence of cockroach blood greatly enhances glial growth (E. A. Howes and P. J. S. Smith in preparation) and also enables isolated neurones from the abdominal and thoracic ganglia to grow under appropriate culture conditions.

This paper describes a method for growing isolated neurones from adult insects and presents preliminary results obtained from calcium-imaging experiments confirming the physiological integrity of the neuronal membranes of these *in vitro* preparations.

Materials and methods

Animals used in all experiments were adult male *Periplaneta americana* (L.), taken from a culture maintained in the Department of Zoology, Cambridge. Animals were kept at a temperature of 29 °C and had free access to food and water.

Cell culture medium

Neurones were cultured in a medium consisting of 5 parts Schneider's *Drosophila* medium and 4 parts Minimum Essential Medium with Hanks' salts and containing 25 mmol l^{-1} Hepes (5+4 medium) obtained from Gibco. The only additives were 1% L-glutamine, 100 i.u. ml^{-1} penicillin and streptomycin, $0.25 \mu\text{g ml}^{-1}$ Fungizone and, in most cases, 0.8% methyl cellulose. The methyl cellulose increased the viscosity of the medium and appeared to improve the chances of the neurones establishing a good contact with the base of the culture dish by reducing fluid movement during transfer of the dishes from laminar flow hood to incubator. It was not, however, essential and neurone growth could occur in its absence.

Preparation of isolated neurones

Cockroaches were anaesthetised by holding them under water for 4 min and were then surface sterilised by rinsing in 70% alcohol for 1 min. Working in a laminar flow cabinet and using sterile dissecting techniques, the animals were pinned out ventral side uppermost on a Sylgard plate. Their legs and the cuticle covering the thorax were removed to expose the meta- and mesothoracic ganglia. These were dissected free of adhering fat and tracheal tissue and placed in a dish containing 5+4 medium with 5 times the normal level of antibiotics and without methyl cellulose. After this rinse the ganglia were transferred to a dish containing 0.5 ml of 0.5–1% collagenase (Sigma, Type 1S) and incubated at 29 °C for 1.5 h. The tissue was then rinsed in two changes of 5+4 medium for a total of 1 h and left in a third change of medium at 29 °C overnight. The incubation in collagenase was critical. Some batches of collagenase appeared to be more active than others, so each new batch was tested at a range of concentrations between 0.1 and 2.0%. With very low concentrations of enzyme the connective sheath failed to soften; at higher concentrations the sheath began to fall apart but neurones removed from such preparations failed to grow. Lengthening the period of collagenase incubation similarly reduced the viability of neurones. Overnight rinsing was not essential but cells at the surface of the ganglia were usually more visible after a prolonged rinse, possibly because residual amounts of collagenase continued a very gentle digestion process. Ganglia were then either placed in dishes coated with a layer of insect blood (see below) and containing 5+4 medium with methyl cellulose and antibiotics or transferred into untreated dishes containing 5+4 medium without methyl cellulose. Cell bodies at the surface of the ganglion were removed using fine insect mounting pins (Watkins and Doncaster) attached to stainless-steel shafts. The outer layers of the ganglion were then teased apart to

reveal more cell bodies; these were similarly removed. Cells in the blood-treated dishes were pushed towards the centre of the dish and left to settle after removal of most of the ganglionic debris with a Pasteur pipette. Cells that were removed and stored in ordinary 5+4 medium in untreated dishes were subsequently pipetted into blood-treated culture dishes containing 5+4 medium with methyl cellulose. The cells were then transferred to a humid (80% relative humidity) incubator chamber at 29°C and left undisturbed for 36 h. Medium was part-changed every 7 days by pipetting off half the medium and replacing it with freshly mixed medium.

Preparation of culture dishes

0.5 ml of 5+4 medium was pipetted into a 60 mm×15 mm organ culture dish (Falcon). The hind leg of an anaesthetised, surface-sterilised cockroach was broken off at the base of the femur and the stump attached to the body was held under the surface of the fluid in the culture dish. The animal was gently squeezed until a drop of blood flowed into the dish. This was allowed to spread and settle for 2 h and was then rinsed three times with fresh 5+4 medium. Dishes were checked under an inverted microscope to ensure that the blood had spread out to cover most of the base with a thin layer of blood cells (not more than 10^4 cells cm^{-2}). The rather viscous haemolymph tended to spread slowly and sometimes formed clots, in which case the dish was rejected. Similarly, dishes that contained a lot of granular material, presumably debris from disintegrating blood cells, were not used. Dishes treated in this way did not show melanization of the blood cells, though this could occur if the dishes were not rinsed thoroughly after the 2 h incubation. In the case of cells grown for Ca^{2+}-imaging, a 22 mm glass coverslip, acid-cleaned and sterilised by soaking in 70% alcohol, was placed in the bottom of a 35 mm×10 mm tissue culture dish before the cockroach was bled into it and the nerve cell bodies were placed on this.

Imaging of intracellular Ca^{2+}

Imaging of free cytosolic calcium in neurones was carried out as described in detail elsewhere (Cheek et al. 1989). Briefly, neurones grown for between 7 and 20 days in vitro were washed in phosphate-buffered saline (PBS) and loaded with 2μmol l^{-1} fura-2 acetoxymethylester for 25 min at room temperature (21–24°C). The cells were washed once in PBS and imaged immediately. Experiments were carried out at room temperature with continuous perfusion of PBS. Saline containing a high level of K^+ (80 mmol l^{-1}) was applied to the cells via a U-tube positioned to within 2 mm of the cell. Using this method the cells were challenged with the stimulus within 1 s of the onset of application. Fluorescent images were obtained by alternate excitation at 340 nm and 380 nm (40 ms each wavelength) using an image-processing system (Imagine, Synoptics Ltd, Cambridge, UK) interfaced to a DEC microvax II microcomputer. The ratio image was obtained at video rate and filtered with a time constant of 200 ms (i.e. 5 ratio images s^{-1}) Determination of $[Ca^{2+}]$ was as described by equation 5 of Grynkiewicz et al. (1985) using a K_d for fura-2/Ca^{2+} of 135 nmol l^{-1}.

Analysis of cell growth

Cells were examined daily using an inverted microscope (Leitz). An infrared source was used to maintain the microscope stage at a temperature of 29°C. Growing cells were photographed using 35 mm Pan F film (Ilford) in a Wild MPS 45 Photoautomat system. Measurements were taken from enlarged prints. The changing branching patterns shown by a group of cells in the course of several days' growth were analysed from photographs by drawing a series of circles of increasing diameter centred upon the cells. The number of neuronal extensions crossing each of these circles was then recorded at each stage of growth.

Results

Cell growth and morphology

Neurones removed from the meso- and metathoracic ganglia ranged in diameter from 20 to 120 μm and were either completely isolated or clustered together in small groups, usually containing no more than half a dozen cells. After dissociation the cells were predominantly rounded, though some cells retained axonal stumps. When such preparations were pipetted into culture dishes not previously treated with insect blood, the cells settled on the base of the dish but failed to attach and did not show any signs of growth. Cells from similar preparations introduced into dishes containing a thin layer of insect blood became loosely attached to the bottom of the dish within 48 h. Outgrowths from such cell bodies occasionally developed within 2 days of plating but more commonly growth was first seen after 5 or 6 days. One dish showed no growth until 22 days after plating, but this lag period was exceptional (Fig. 1). Cells grown on blood-coated glass coverslips showed a similar time course of neurite extension.

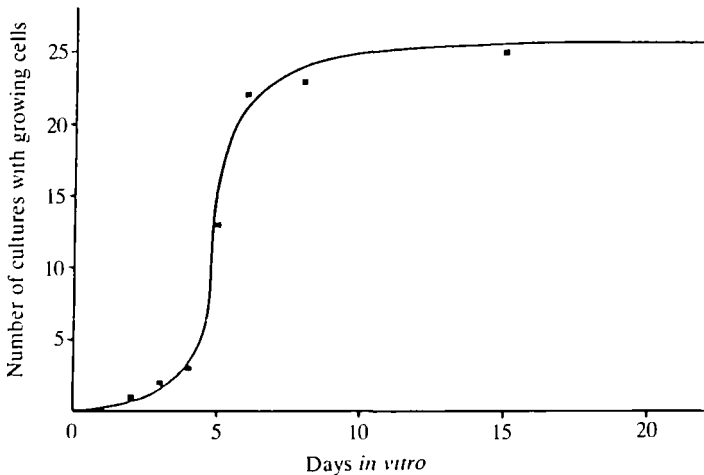Many of the dissociated cells failed to grow; in a sample of six dishes, each

Fig. 1. A graph showing the time of first appearance of growing neurones in each of a sample of 26 culture dishes containing cells isolated from two thoracic ganglia per dish.
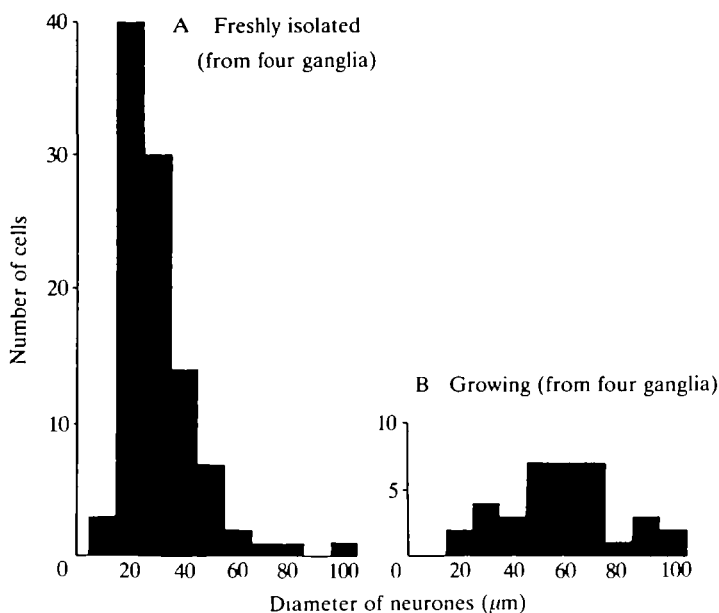
Fig. 2. Histograms to show the range of size of isolated neurones that develop outgrowths *in vitro*. (A) The number and size range (measured as largest diameter) of neurones isolated from two meso- and two metathoracic ganglia. (B) The number and size range of growing neurones from cells isolated from two meso- and two metathoracic ganglia.

containing 40 cells taken from one meso- and one metathoracic ganglion, $23.75 \pm 12.2 \%$ (s.d.) of the neurones grew. The most successful dish contained 16 growing neurones, the least successful contained just three. Of the neurones that did grow, a high proportion were relatively large cells. Fig. 2A shows the numbers of cells of different sizes obtained from four ganglia and measured directly after dissociation. Most cells were small, with diameters falling within the range $20-50 \mu m$. In contrast, the range of sizes of growing cells from another four ganglia is shown in Fig. 2B. Cells of $50 \mu m$ or more in diameter were most successful in developing extensions and, given the small proportion of dissociated cells larger than $80 \mu m$, growth in the size range $80-110 \mu m$ was very successful.

Growing cells showed a variety of morphologies (Fig. 3). In some cases, a number of processes radiated from the cell body (Fig. 3A) and formed complicated networks as they elongated (Fig. 3B); more commonly one or two main processes grew (Fig. 3C,D). These usually developed secondary branches to a greater or lesser extent (Fig. 3E,F). In a few cases, the growing point bifurcated to form two branches of equal size (Fig. 4A). Growing cells with axonal stumps usually developed sprouts from the end of the severed stump (Fig. 4B). With phase-contrast microscopy some neurites had a 'beaded' appearance, caused by the presence of phase-bright swellings on the extending processes. These were not common to all neurites growing in a dish (Fig. 4C) and so were not caused by

deteriorating culture conditions. 'Beaded' neurites grew as extensively as smooth ones and it was noted that branching was often associated with the swellings.

Rates of growth of the cell extensions varied, the fastest recorded was 344 μm in a single day (equivalent to 14.3 μm h^{-1}). In a group of eight cells observed for 15 days after the initiation of growth, the average rate of growth was 3.9±0.48 μm h^{-1}.

The pattern of growth from a small clump of cells was examined in detail over a period of 21 days (Fig. 5). There was no sign of growth for the first 5 days *in vitro* and then extensions from three cells developed on day 6. By day 11 the number of cell extensions had risen to eight and this increased to 12 by day 14. Over this period the processes thickened and lengthened, some developed side branches, and further branching occurred at the growing tips. Growth continued during the next 7 days and the developing network exhibited considerable plasticity, with new side branches appearing while existing branches disappeared. In some cases whole processes retracted. The longest process from this group covered a distance of 1875 μm in 19 days of growth (21 days *in vitro*), after which the culture was used in calcium-imaging experiments. Growing processes radiated from the central clump of cells but the direction of growth could alter sharply when the axon encountered an irregularity, such as a scratch on the surface of the culture dish or a clump of haemocytic debris. The axons often appeared to become firmly anchored at these points. The whole axonal network was closely apposed to the surface of the culture dish and was firmly attached to it, whereas the neuronal cell bodies were much more loosely held and could move in relation to the surface of the dish, presumably pulled along by the growing axon.

The pattern of branching shown by the group of neurones illustrated in Fig. 5 was analysed and the results are summarised in Fig. 6. At the early stages of growth, branching was, of neccessity, confined to the area immediately adjacent to the cell bodies. As the growing processes elongated, branching was seen both close to the cell body and further along the extending processes. The number of branches occurring within 30 μm of the cell bodies fluctuated but was highest at 12 and 14 days *in vitro*. The number of branches in this area then declined and by 19 days *in vitro* the peak of branch formation appeared 40 μm distant from the cell bodies. Two days later, by 21 days *in vitro*, this peak had shifted to 70 μm from the centre and an increasing number of new branches appeared near the peripheral ends of the main processes. This analysis illustrates the plasticity of the growing neurites, whose side branches can form and retract in the space of a few days.

Neurones survived in culture for periods of up to 6 weeks. After this, signs of deterioration became apparent, with axons developing a very beaded appearance and becoming increasingly attenuated. The cells grown on haemolymph-coated glass coverslips survived less well than those grown on similarly coated plastic and often began to deteriorate after 2–3 weeks *in vitro*.

Calcium imaging

Calcium ions, acting as second messengers, play an important role in controlling
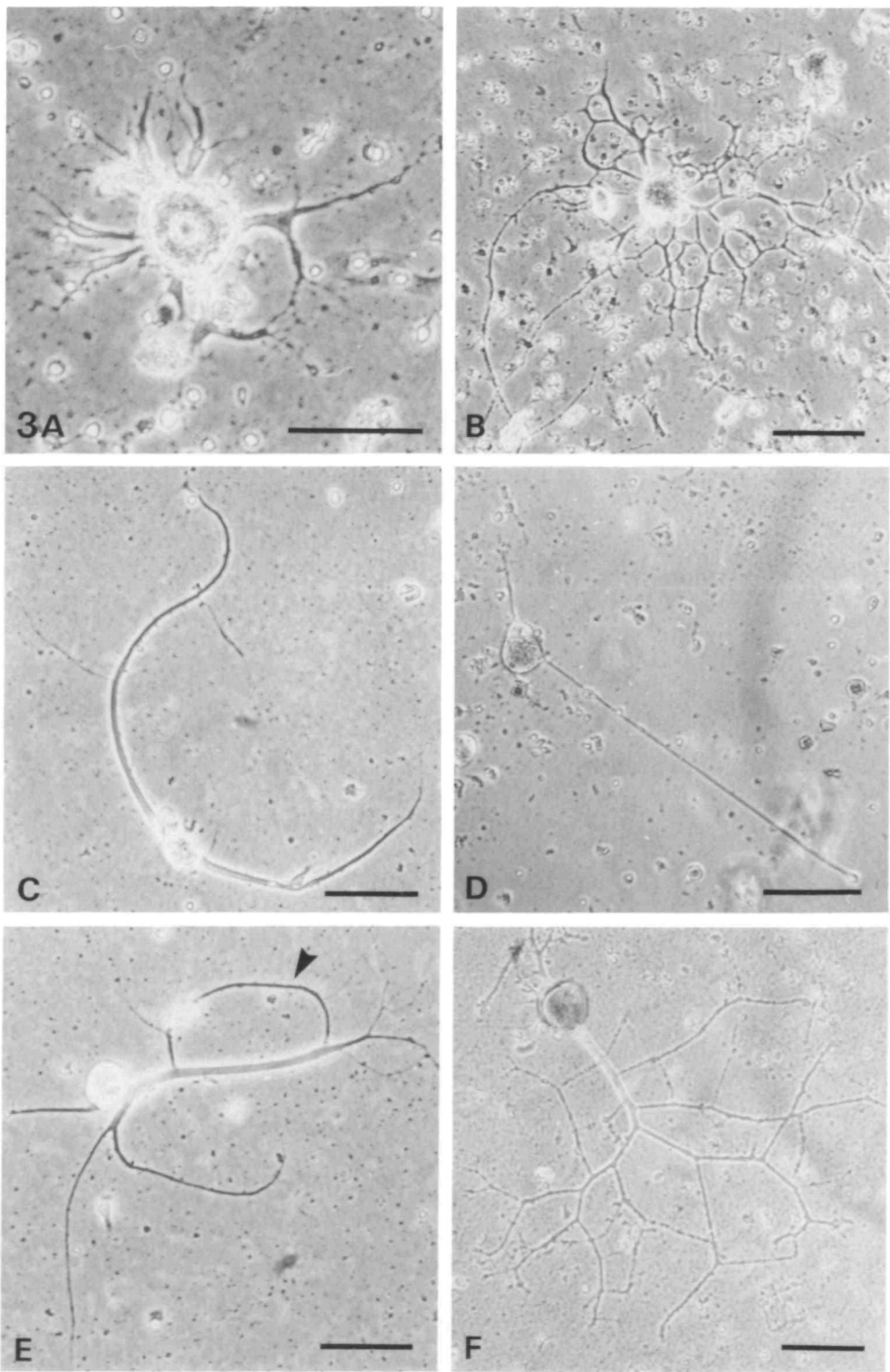
Fig. 3. Phase-contrast light micrographs showing outgrowths from neurones isolated from thoracic ganglia and maintained *in vitro* on a substratum derived from insect blood. (A) A single cell showing multiple process outgrowth after 5 days in culture. (B) A neurone after 16 days in culture surrounded by a network of processes. (C) An isolated neurone showing bipolar growth after 8 days in culture. This neurone has processes that are characteristically curved and have very few branches. (D) A neurone from a 12-day-old culture showing a single main process that is both straight and unbranched. (E) Branching pattern of a neurone from a 10-day-old culture showing curving branches some of which turn back towards the main trunk (arrowhead). (F) Branching pattern of an isolated neurone after 25 days in culture. Here branches have radiated from the main neurite and further secondary branches have developed to form a network on one side of the cell (in contrast to the network surrounding the cell in Fig. 3B, where the branching pattern develops from a number of processes). Scale bars, 100 μm.

neuronal function (Miller, 1988, and see Discussion). Therefore, we have used the fluorescent Ca^{2+} indicator dye fura-2 to assess the ability of the cultured neurones to regulate their $[Ca^{2+}]_i$ in response to a depolarizing stimulus. Fig. 7A shows that, during perfusion with PBS of the cell at rest, low levels of intracellular Ca^{2+} were distributed throughout the growing cell. These resting levels of $[Ca^{2+}]_i$ had a mean value of $46 \, nmol \, l^{-1}$. A 3s pulse of $80 \, mmol \, l^{-1} \, K^+$ to depolarize the cell membrane caused an increase in the $[Ca^{2+}]_i$ of the axon processes; this rose to a peak of $711 \, nmol \, l^{-1}$ by the end of the pulse (Fig. 7B). The rise began to decay within 1 min (Fig. 7C) and by 6 min $[Ca^{2+}]_i$ had returned to the resting level (Fig. 7D). This result probably indicates the existence of voltage-dependent Ca^{2+} channels on the axon processes, although the possibility that some of the rise in $[Ca^{2+}]_i$ was due to Ca^{2+}-induced release of Ca^{2+} from an internal store, as has been suggested to occur in bullfrog sympathetic neurones (Lipscombe *et al.* 1988; Tsien, 1988), cannot be ruled out. The failure of the central regions of the soma and the large process to match the pseudocolour change of the rest of the cell is an example of photometric inaccuracy, due here to the high camera gain required to see thin processes and the consequent overloading on the thickest and brightest areas of the cell.

Discussion

Cockroach neurones can be isolated from the ganglia of adult insects following collagenase treatment. These cells, deprived of their axonal projections, can be induced to develop new axonal growth when maintained *in vitro* in the presence of cockroach blood. Thus, fully developed cells with an adult phenotype can, under suitable conditions, be grown *in vitro*.

The number of cells isolated from each ganglion is small compared to the total number of neurones present, estimated to be approximately 500 motoneurones and 1500 interneurones (Pearson, 1977). Since the technique employed involved the removal of cells that were readily visible we have tended to select the largest cells and those that lie in well-defined groups with conspicuous axonal tracts
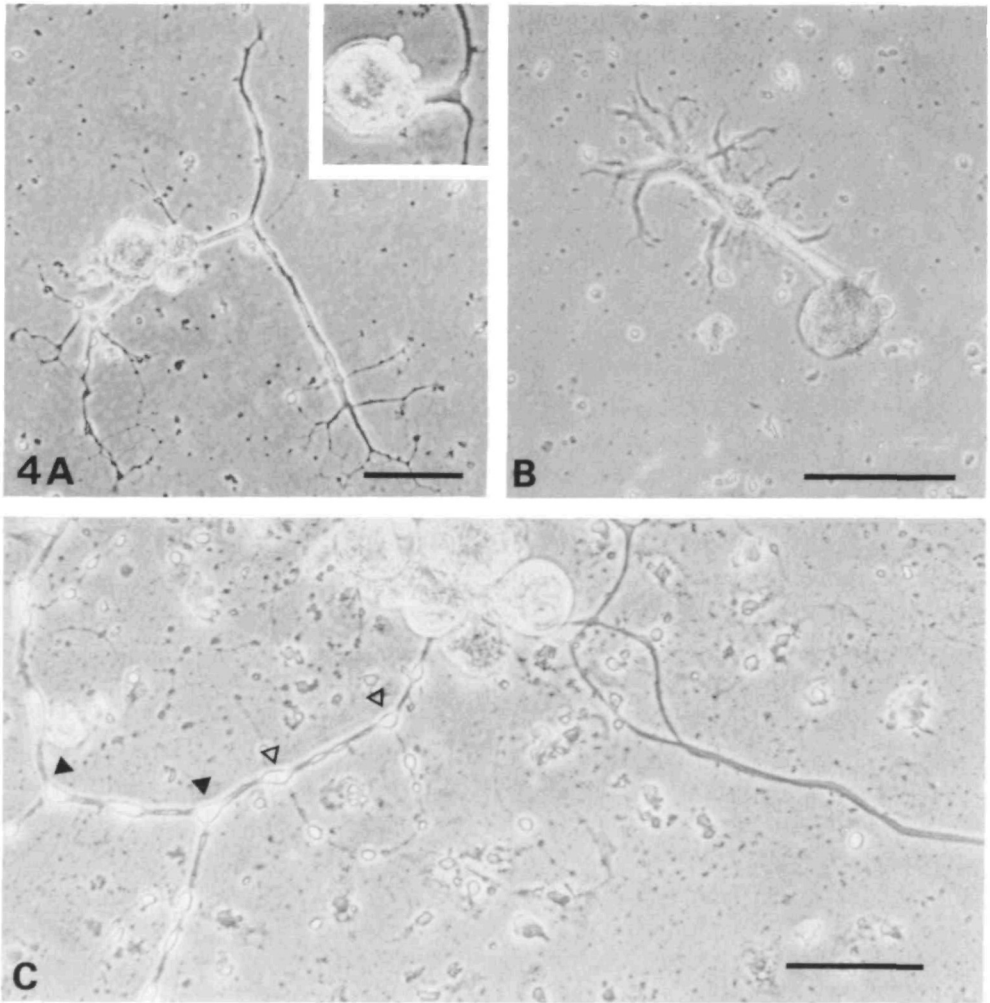
Fig. 4. Phase-contrast light micrographs of (A) a small cluster of neurones from which a bifurcating process has grown out. Inset: a higher magnification view of an isolated cell showing a similar morphology. (B) An isolated neurone removed with a short stump of axon from the metathoracic ganglion showing the sprouting that occurs at the severed axon tip after 5 days in culture. (C) Processes growing out from a clump of neurones. One of the processes is smooth, the other has a markedly 'beaded' appearance. Branches are often associated with the 'beads' (arrowheads) and, in one case, a branch appears to emerge from one bead and re-enter another (open arrowheads). Scale bars, 100 μm.

leading from them. Such cells are found near the base of nerve trunks entering the ganglia (Cohen and Jacklet, 1967; Gregory, 1974). In addition, our results show that the larger neurones seem better able to survive in culture than smaller cells, so that cells 50 μm or more in diameter are selected by the culturing technique.

A variety of morphologies is exhibited by such cells, so that while some cultured
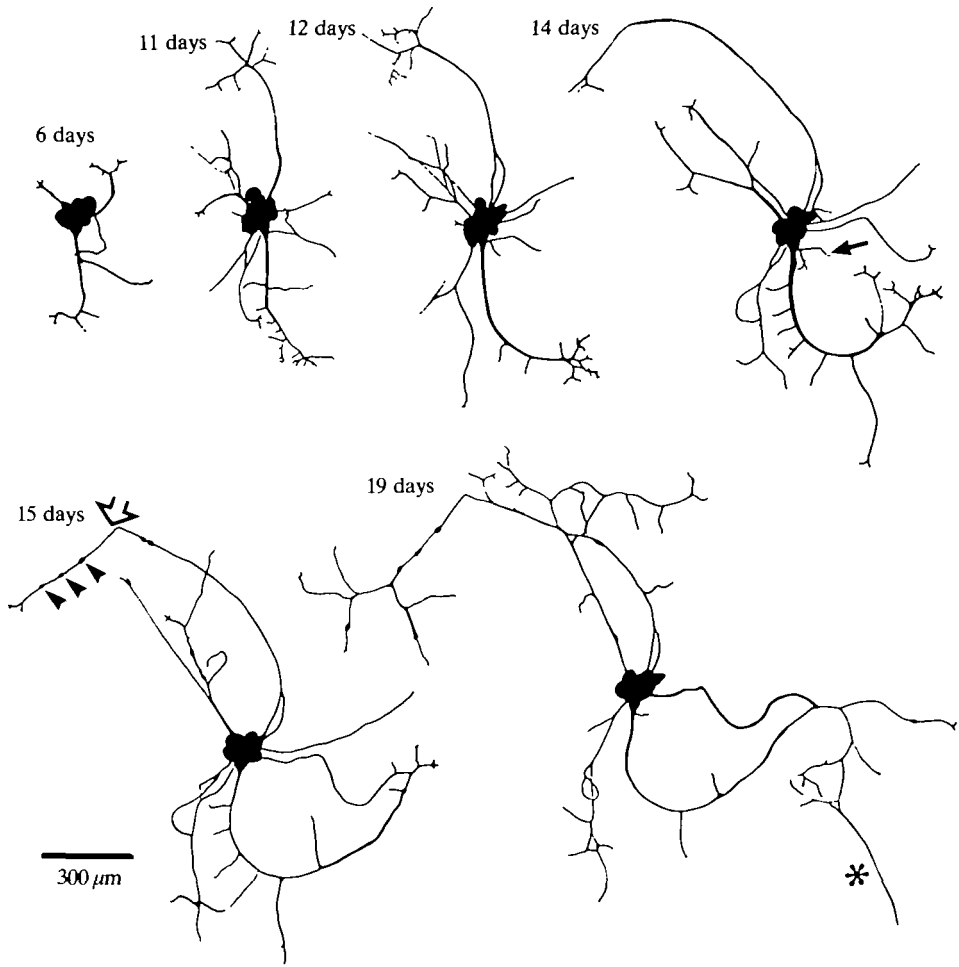
Fig. 5. A series of tracings of the processes emerging from a clump of cells between 6 and 19 days *in vitro* to illustrate the changing shape of outgrowth. The development of an increasingly complex network of processes can be followed. Some processes form only to disappear, e.g. the processes arrowed at 14 days are absent by 15 days, while others steadily elongate throughout the period of observation. In some cases the growth of a process may be altered by the physical conditions obtaining in the culture dish, e.g. the sharp turn displayed by one of the processes (open arrow) coincides with a scratch on the base of dish. In this growth sequence 'beads' first began to appear on some of the outgrowths after 15 days (arrowheads). The process marked with an asterisk was derived from an adjoining group of neurones.

neurones develop a single extension, others appear to be bipolar and yet others develop quite complex networks with numerous processes arising from a single cell. In some cases this diversity of growth *in vitro* may reflect the morphology of the cell *in vivo*. Prominent among the cells visible on the dorsal surface of the ganglion after collagenase treatment is a small group of large cells (50–60 μm)
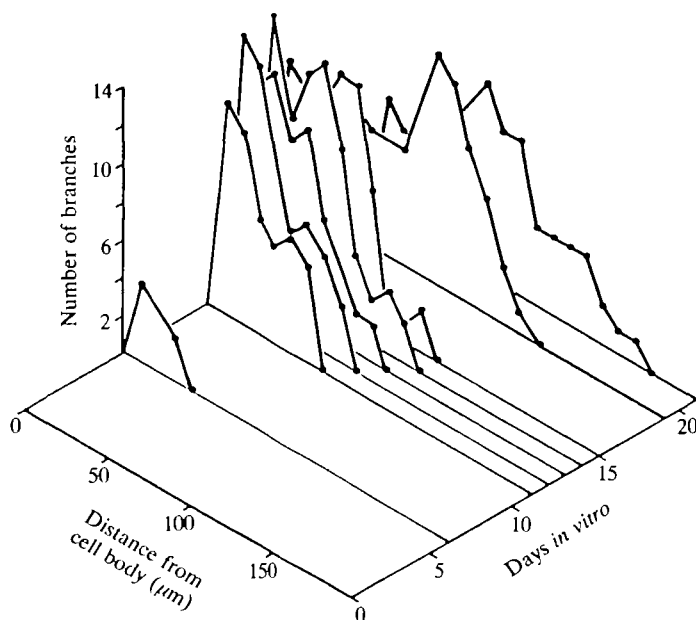
Fig. 6. Graph showing the appearance of branches and the change of their distribution with time in the group of cells shown in Fig. 5. The number of branches was estimated by counting the number of processes crossing a series of circles of increasing radius (in 10 μm steps) drawn around the central cell clump. As processes extended, the main area of branching moved away from the area of the cell body and occurred further towards the extending tip.

situated along the midline. These correspond in position and size to the dorsal unpaired medial (DUM) cells whose location and morphology has been described (Crossman *et al.* 1971). A characteristic of DUM cells is the bifurcation of the cell process a short distance from the cell body to give a bilaterally symmetrical pattern of growth. This is reminiscent of the growth pattern shown by some cells *in vitro* (e.g. Fig. 4A). Typically, neurones in the adult ganglia are monopolar, though some sensory neurones may be bipolar. The appearance of neurones with multiple outgrowths has not been described in the intact ganglia and their appearance in culture may reflect random growth in the absence of any guidance cues or target tissues.

The neurones isolated by this technique are not identified, but it would seem possible to remove large cells in obvious locations that have been described physiologically, such as the DUM cells, and to culture these individually. Such preparations could prove particularly valuable in studying physiological responses of single cells in the absence of input from other neural elements.

Changes in $[Ca^{2+}]_i$ within neurones play a pivotal role in the control of many neuronal functions such as excitability (Hagiwara and Byerly, 1983), synaptic transmitter release (Smith and Augustine, 1988; Delaney and Zucker, 1990), growth cone motility (Connor, 1986) and neurite extension (Silver *et al.* 1990, but
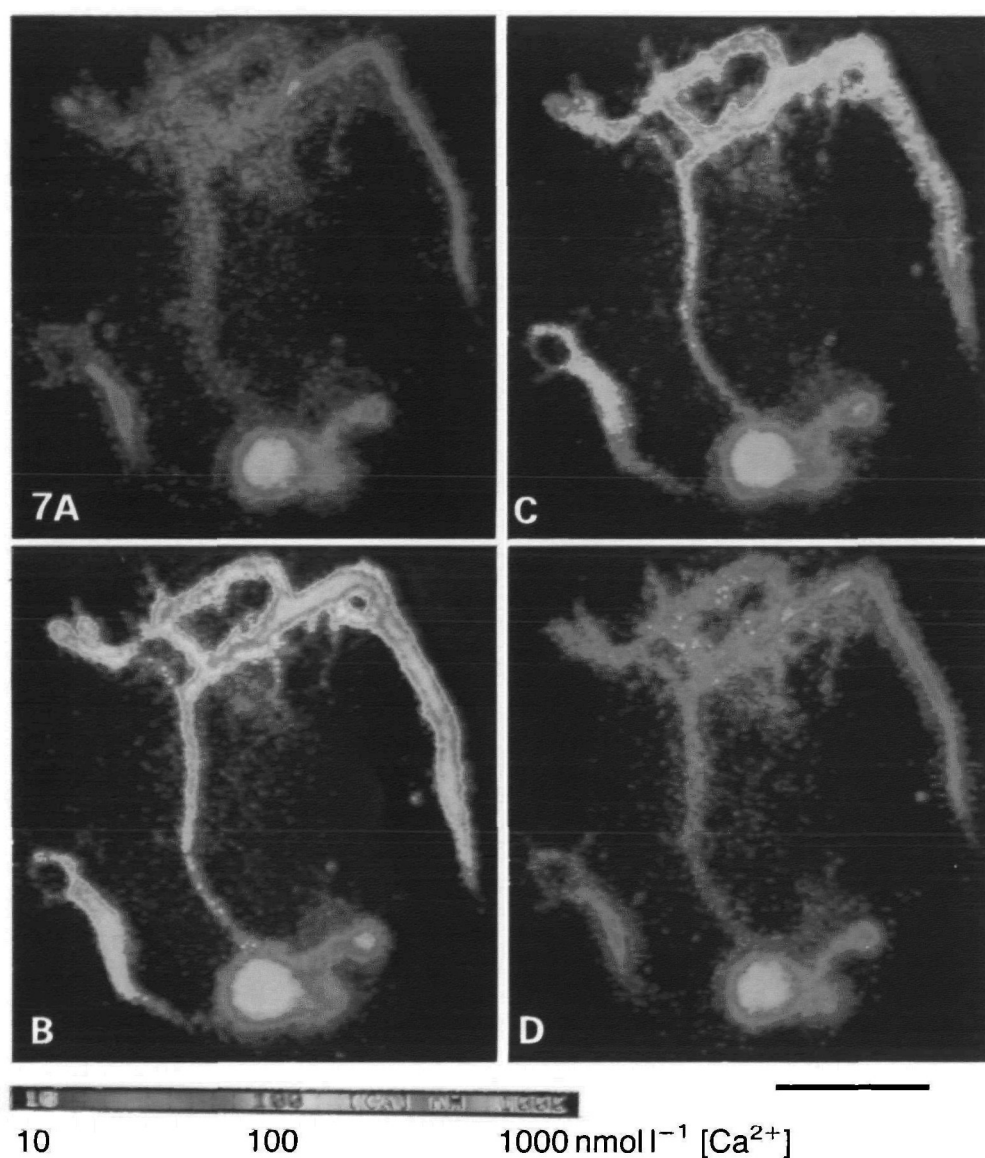
Fig. 7. Fluorescence ratio images of the intracellular levels of free cytosolic Ca^{2+} in a cultured neurone loaded with fura-2 and the changes induced by K^+ depolarization. (A) Basal levels of $[Ca^{2+}]_i$ in the resting cell. (B) $[Ca^{2+}]_i$ 3 s after the perfusion of 80 mmol l^{-1} K^+ over the neurone, showing a dramatic rise. (C) $[Ca^{2+}]_i$ 1 min after perfusion with K^+, showing a reduction of the effect. (D) 6 min after K^+ depolarization $[Ca^{2+}]_i$ has returned to resting levels. Scale bar, 100 μm.

see Tolkovsky *et al.* 1990, for an alternative view). In the present study we used fura-2 imaging to assess the ability of cultured cockroach neurones to regulate their $[Ca^{2+}]_i$ in response to depolarization with high-K^+ saline. These experiments showed that the cockroach neurones isolated by this technique were able to respond with a rise in $[Ca^{2+}]_i$ following chemical depolarization. That $[Ca^{2+}]_i$ was elevated in the dendrites suggests the presence of voltage-operated Ca^{2+} channels in the plasma membrane of this area of the cell. Similarly localized voltage-operated Ca^{2+} channels have also been reported in cultured rat Purkinje neurones (Hockberger *et al.* 1989). Preliminary experiments have indicated that these channels are also present on the soma of cockroach neurones. Cultures of frog sympathetic neurones (Lipscombe *et al.* 1988) and rat dorsal root ganglion neurones (Thayer *et al.* 1988) also have voltage-operated Ca^{2+} channels both on the dendrites and on the soma. The effects that various neuromodulatory agents have on the Ca^{2+} signal triggered by K^+-induced depolarization in cockroach neurones is currently under investigation.

The factor, or factors, in the blood that enable neurones to grow *in vitro* have not been identified. From our work on glial cell culturing it is obvious that attachment factors play an important part in the growth process (Howes *et al.* 1989) and it seems probable that an attachment molecule is involved in neuronal growth. However, preliminary experiments with fibronectin, laminin, polyl-L-lysine and Matrigel did not lead to neuronal growth (E. A. Howes, personal observation), though Pinnock and Sattelle (1987) reported that neurones stuck for extended periods to concanavalin-A-treated coverslips. Glycoproteins have been implicated in the guidance of growth cones and three different membrane-associated glycoproteins, Fasciclins I–III, have been identified in insect nervous systems as potential molecular modulators of growth (Bastiani *et al.* 1987; Patel *et al.* 1987). Nicholls *et al.* (1990) have demonstrated that different attachment molecules can affect both the morphology and the distribution of Ca^{2+} channels in leech Retzius cells *in vitro*. Similar effects might be expected in the insect CNS, and this preparation offers considerable promise as a system in which the effects of attachment molecules, neurotransmitters and growth factors on neuronal regeneration and morphological plasticity can be directly observed.

References

BASTIANI, M. J., HARRELSON, A. L., SNOW, P. M. AND GOODMAN, C. S. (1987). Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. *Cell* **48**, 745–755.

BEADLE, D. J. AND HICKS, D. (1985). Insect nerve culture. In *Comprehensive Insect Physiology Biochemistry and Pharmacology*, vol. 5 (ed. G. A. Kerkut and L. I. Gilbert), pp. 213–298. Oxford: Pergamon Press.

CHEEK, T. R., JACKSON, T. R., O'SULLIVAN, A. J., MORETON, R. B., BERRIDGE, M. J. AND

Burgoyne, R. D. (1989). Simultaneous measurements of cytosolic calcium and secretion in single bovine adrenal chromaffin cells by fluorescent imaging of fura-2 in cocultured cells. *J. Cell Biol.* **109**, 1219–1227.

Chen, J. S. and Levi-Montalcini, R. (1970). Axonal growth from insect neurones in glia-free cultures. *Proc. natn. Acad. Sci. U.S.A.* **66**, 32–39.

Cohen, M. J. and Jacklet, J. W. (1967). The functional organization of motor neurons in an insect ganglion. *Phil. Trans. R. Soc. Ser.* B **252**, 561–572.

Connor, J. A. (1986). Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian central nervous system cells. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6179–6183.

Crossman, A. R., Kerkut, G. A., Pitman, R. M. and Walker, R. J. (1971). Electrically excitable nerve cell bodies in the central ganglia of two insect species *Periplaneta americana* and *Schistocerca gregaria*. Investigations of cell geometry and morphology by intracellular dye injection. *Comp. Biochem. Physiol.* A**40**, 579–594.

Dagan, D. and Levitan, I. B. (1981). Isolated identified *Aplysia* neurons in cell culture. *J. Neurosci.* **1**, 736–740.

Delaney, K. R. and Zucker, R. S. (1990). Calcium released by photolysis of DM-nitrophen stimulates transmitter release at squid giant synapse. *J. Physiol., Lond.* **426**, 473–498.

Giles, D. P. and Usherwood, P. N. R. (1985). Locust nymphal neurones in culture: a new technique for studying the physiology and pharmacology of insect central neurones. *Comp. Biochem. Physiol* **80C**, 53–59.

Graf, R. A. and Cooke, I. M. (1990). Primary culture of crustacean stomatogastric ganglion neurones in a defined medium. *J. exp. Biol.* **149**, 521–525.

Gregory, G. E. (1974). Neuroanatomy of the mesothoracic ganglion of the cockroach *Periplaneta americana* (L.). I. The roots of the peripheral nerves. *Phil. Trans. R. Soc. Ser.* B **267**, 421–465.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J. biol. Chem.* **260**, 3440–3450.

Hagiwara, S. and Byerly, L. (1983). The calcium channel. *Trends Neurosci.* **6**, 189–193.

Harrison, J. B., Leech, C. A., Katz, J. and Sattelle, J. B. (1990). Embryonic and adult neurones of the housefly (*Musca domestica*) in culture. *Tissue & Cell* **22**, 337–347.

Hockberger, P. E., Tseng, H.-Y. and Connor, J. A. (1989). Fura-2 measurements of cultured rat Purkinje neurons show dendritic localization of Ca^{2+} influx. *J. Neurosci.* **9**, 2272–2284.

Howes, E. A., Chain, B. M., Smith, P. J. S. and Treherne, J. E. (1987). Blood cells contribute to glial repair in an insect. *Tissue & Cell* **19**, 877–880.

Howes, E. A., Smith, P. J. S. and Treherne, J. E. (1989). Adult insect glial culture: activation, substrate effects and proliferation. *Tissue & Cell* **21**, 759–772.

Lipscombe, D., Madison, D. V., Poenie, M., Reuter, H., Tsien, R. Y. and Tsien, R. W. (1988). Spatial distribution of calcium channels and cytosolic calcium transients in growth cones and cell bodies of sympathetic neurons. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2398–2402.

Miller, R. J. (1988). Calcium signalling in neurons. *Trends Neurosci.* **11**, 415–418.

Nicholls, J. G., Liu, Y., Payton, B. W. and Kuffler, D. P. (1990). The specificity of synapse formation by identified leech neurones in cultures. *J. exp. Biol.* **153**, 141–154.

Patel, N. H., Snow, P. M. and Goodman, C. S. (1987). Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* **48**, 975–988.

Pearson, K. G. (1977). Interneurons in the ventral cord of insects. In *Identified Neurons and Behaviour of Arthropods* (ed. G. Hoyle), pp. 329–338. New York, London: Plenum Press.

Pinnock, R. D. and Sattelle, D. B. (1987). Dissociation and maintenance *in vitro* of neurones from adult cockroach (*Periplaneta americana*) and housefly (*Musca domestica*). *J. Neurosci. Methods* **20**, 195–202.

Ready, D. F. and Nicholls, J. G. (1979). Identified neurones isolated from leech CNS make selective connections in culture. *Nature* **281**, 67–69.

Schacher, S. and Proshansky, E. (1983). Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J. Neurosci.* **3**, 2403–2413.

SESHAN, K. R. AND LEVI-MONTALCINI, R. (1973). Neuronal properties of nymphal and adult insect neurosecretory cells *in vitro*. *Science* **182**, 291–293.

SILVER, A., LAMB, A. G. AND BOLSOVER, S. R. (1990). Calcium hotspots caused by L-channel clustering promote morphological changes in neuronal growth cones. *Nature* **343**, 751–754.

SMITH, P. J. S., HOWES, E. A., LEECH, C. A. AND TREHERNE, J. E. (1986). Haemocyte involvement in the repair of the insect central nervous system after selective glial disruption. *Cell. Tiss. Res.* **243**, 367–374.

SMITH, P. J. S., HOWES, E. A. AND TREHERNE, J. E. (1990). Cell proliferation in the repairing adult insect central nervous system: incorporation of the thymidine analogue 5-bromo-2-deoxyuridine *in vivo*. *J. Cell Sci.* **95**, 599–604.

SMITH, S. J. AND AUGUSTINE, G. J. (1988). Calcium ions, active zones and synaptic transmitter release. *Trends Neurosci.* **11**, 458–464.

THAYER, S. A., PERNEY, T. M. AND MILLER, R. J. (1988). Regulation of calcium homeostasis in sensory neurones by bradykinin. *J. Neurosci.* **8**, 4089–4097.

THOMAS, W. E., JORDAN, F. L. AND TOWNSEL, J. G. (1987). The status of the study of invertebrate neurons in tissue culture – Phylum Arthropoda. *Comp. Biochem. Physiol.* **87A**, 215–222.

TOLKOVSKY, A. M., WALKER, A. E., MURRELL, R. D. AND SUIDAN, H. S. (1990). Ca^{2+} transients are not required as signals for long term neurite outgrowth from cultured sympathetic neurons. *J. Cell Biol.* **110**, 1295–1306.

TSIEN, R. Y. (1988). Fluorescence measurements and photochemical manipulation of cytosolic free calcium. *Trends Neurosci.* **11**, 419–424.

WHITE, K. AND KANKEL, D. R. (1978). Patterns of cell division and cell movement in the formation of the imaginal nervous system in *Drosophila melanogaster*. *Devl Biol.* **65**, 296–321.

WU, C.-F., SUZUKI, N. AND POO, M.-M. (1983). Dissociated neurons from normal and mutant *Drosophila* larval central nervous system in cell culture. *J. Neurosci.* **3**, 1888–1899.